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PATENT APPLICATION
Docket No. 15892.9

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of		)
	William Richard Cross et al.	)
Serial No.:	10/522,371	) Art Unit
Filed:	January 25, 2005	) 1657
Confirmation No.:	1386	)
For:	BIOMIMETIC UROTHELIUM	)
Examiner:	Laura J. Schuberg	)
Customer No.:	22913	)

DECLARATION OF JENNIFER SOUTHGATE, PH.D. UNDER 37 C.F.R. 1.131 & 1.132

Mail Stop AMENDMENT
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Dear Sir:

I, Jennifer Southgate, Ph.D. hereby declare as follows:

1. I am personally knowledgeable of the facts stated herein.

2. I am a co-inventor of U.S. Patent Application Serial No. 10/522,371 ("Subject Application").

3. I am an employee of The University of York, which has ownership of the Subject Application via assignment, and thereby I have a personal interest in the Subject Application.

4. I have significant experience in the art of human urothelium and the preparation of ex vivo human urothelium tissue and the stratification and differentiation thereof as applied in the Subject Application which is currently under examination.

5. I have reviewed and understand the Subject Application and the Cross and Zhang references. In fact, I co-authored the Cross reference.

6. I have reviewed the response to the Office Action being filed herewith, and attest that the human urothelial differentiation method as presently claimed is not obvious over the art of record. This is because none of the references, separately or combined, indicates that incubating urothelium cells (isolated from a human body) in a serum-containing medium, and then disrupting the cells by redispersing them prior to transfer into a fresh serum-containing medium, could produce stratified, terminally-differentiated human urothelium. The ability to obtain stratified, terminally-differentiated human urothelium after the redispersal step is surprising and unexpected because such redispersal (into a second medium containing serum) is not a routine or traditional procedure for obtaining stratified, terminally-differentiated tissue (such as human urothelium or indeed other differentiated epithelia). The morphology and phenotype obtained from immunocytochemistry and transmission electron microscopy coupled with the transepithelial electrical resistance provided evidence that stratified, terminally-differentiated tissue had been obtained, for the first time using an ex-vivo method as now claimed.

7. The invention is surprising and unexpected because:

a) following a first incubation of cells (e.g. urothelial cells) in serum it is counterintuitive to redisperse the cells, prior to a further incubation in serum, in order to obtain a stratified, terminally-differentiated tissue; the skilled addressee would seek to maintain any structure from the first incubation in serum, rather than disrupting it by redispersal; and

b) the art clearly teaches that use of serum and resdispersal of cells is routine procedure for the propagation of cells whilst, on the other hand, maintenance of any tissue structure is a routine procedure for the differentiation/stratification of cells (please see Appendix B for a summary of the art in this respect). Thus the skilled addressee, when seeking to obtain a stratified, terminally-

differentiated human urothelium would be motivated by the art to exclude serum, and to maintain preliminary structure (rather than disrupting it by redispersing cells), during the method of preparation i.e. exactly the opposite of what we have done in the present invention. To arrive at our invention we went against the technical teachings in the art that were available to the skilled addressee and we were surprised to obtain the excellent results that we have presented.

8. Accordingly, it is surprising and unexpected that we were able to obtain stratified, terminally-differentiated human urothelium in which urothelial cells, isolated from the human body and propagated by culture in serum-free nutrient medium are transferred to a first nutrient differentiation medium containing serum and then redispersed by passage before being added to a second nutrient differentiation medium containing serum to form said urothelium.

9. I attest that the immunocytochemistry and transmission electron microscopy experiments that were conducted that provided the morphology and phenotype characterization of stratified, terminally-differentiated human urothelium were performed prior to the publication of Cross et al. (Biochemical Society Transactions 2001, which is cited in the IDS). I also attest that the transepithelial electrical resistance experiments were conducted before the publication of Cross et al., which when combined with the morphology and phenotype data characterized the cells to be stratified, terminally-differentiated human urothelium. Therefore, the conception of the invention of the claimed method occurred prior to Cross et al. being available to the public on December 18, 2001.

10. I attest that the experimental method and data that provided the characterization that the cells were indeed stratified, terminally-differentiated human urothelium is provided in Appendix A below.

11. I attest that the listing of inventors of the instant patent application is correct and there are no other inventors that contributed to the conception of the invention as claimed.

12. I declare further that all statements made herein of my own knowledge are true and that all statements are made on information and belief are believed to be true; and further that these

statements were made with the knowledge that willful, false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful, false statements may jeopardize the validity of the application or any patent issuing thereon.

Signed this 16th day of June 2010.

JENNIFER SOUTHGATE, PH.D.

APPENDIX A

Experimental detail

Normal human urothelial (NHU) cell cultures were established and propagated in standard growth conditions in low calcium (0.09 mM) keratinocyte serum free growth medium containing bovine pituitary extract, epidermal growth factor and supplemented with 30ng/ml cholera toxin (labeled KSFM).

For passage, just-confluent cultures were incubated in 5ml of 0.1% (w/v) EDTA in PBS for 5 minutes at 37°C, followed by 1ml 0.25% (w/v) trypsin in PBS containing 0.02% (w/v) EDTA until cells were released. Cells were suspended in KSFM containing 1.5mg/ml of soybean trypsin inhibitor and centrifuged at 250g for 5 minutes. The cell pellet was resuspended in KSFM and reseeded into two Primaria flasks. After 24 hours, the medium was changed with one (control) flask maintained in KSFM and the other maintained in KSFM supplemented with 5% fetal bovine serum (FBS).

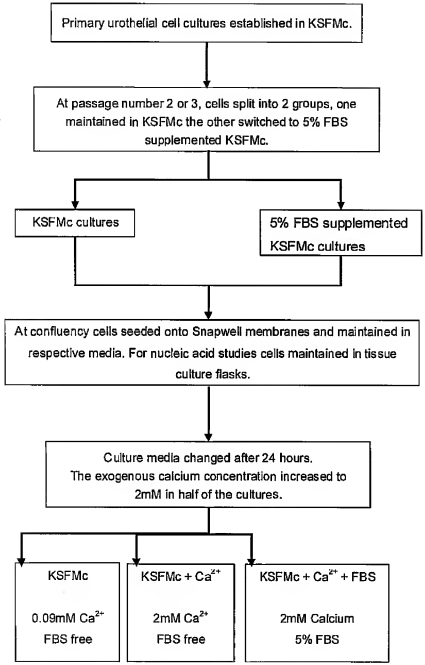
After 5 days, cultures were harvested (as above) and seeded in their respective media onto Snapwell membranes at $1x10^6$ cells/cm^2. After 24 hours, the medium in half of the control cultures and all of the FBS-supplemented cultures was increased to 2mM. Medium was replaced after 24 hours and subsequently on alternate days for all experiments. Cultures were left for a further 6 days before analysis.

The morphology and phenotype of the urothelial cultures was assessed by immunocytochemistry and transmission electron microscopy. Ionic permeability was determined by measuring the transepithelial electrical resistance (TER) using a modified Ussing chamber and electronic volt-ohmmeter. Transurothelial sodium transport was delineated using appropriate ion channel inhibitors. Urothelial permeability to urea and water was determined by a radioisotope tracer technique.

Results

When cultured in low calcium serum-free medium urothelial cells formed monolayers. Phenotypically the cells expressed markers characteristic of basal/intermediate cells in vivo and did not form intercellular tight junctions or show attributes of barrier function (TER 16.6 ± 2.1 $\Omega.cm^2$; permeability for urea 11.8 ± 4.8 $x10^{-5}$ cm/s and water 4.7 ± 3.1 $x10^{-4}$ cm/s). Increasing the exogenous calcium concentration resulted in urothelial stratification, expression of tight

junctions and a significant increase in transepithelial electrical resistance (TER 43.0 ± 3.7 $\Omega.cm^2$, $p<0.01$) and decrease in urea and water permeability (urea 3.0 ± 1.4 $x10^{-5}$ cm/s, water 2.2 ± 0.8 $x10^{-4}$ cm/s, $p<0.01$). The cultures grown in serum-supplemented medium and physiological calcium were stratified and showed evidence of uroplakin expression and tight junction formation. In addition, they exhibited the greatest electrical resistance (TER 2567.1 ± 475 $\Omega.cm^2$, $p<0.01$), a functional amiloride-sensitive sodium transport system, plus urea and water permeabilities of 12.4 ± 7.6 $x10^{-5}$ cm/s and 5.5 ± 4.8 $x10^{-4}$ cm/s, respectively.


Primary urothelial cell cultures established in KSFMc.
At passage number 2 or 3, cells split into 2 groups, one maintained in KSFMc the other switched to 5% FBS supplemented KSFMc.
KSFMc cultures
5% FBS supplemented KSFMc cultures
At confluency cells seeded onto Snapwell membranes and maintained in respective media. For nucleic acid studies cells maintained in tissue culture flasks.
Culture media changed after 24 hours.
The exogenous calcium concentration increased to 2mM in half of the cultures.
KSFMc
0.09mM Ca2+
FBS free
KSFMc + Ca2+
2mM Ca2+
FBS free
KSFMc + Ca2+ + FBS
2mM Calcium
5% FBS

Culture Medium	Morphology Phenotype	Electrophysiology		Permeability				
		TER ($\Omega.cm^2 \pm SEM$) N=6	Na Transport	FITC-Dextran (MW) 4400 / 9500 (μg/ml ± SD)* N=4	[^{14}C]Urea ($\times 10^{-6}$ cm/s) N=4	[^{3}H]Water ($\times 10^{-6}$ cm/s)
KSFM (0.09 mM Ca)	Monolayer. No tight junction. Resembles basal/intermediate cells in vivo.	16.6 ± 2.1	Not assessed.	50.8 ± 30.5	26.8 ± 15.6	11.8 ± 4.8	4.7 ± 3.1
2 mM Ca KSFM	Stratified. Tight junctions. Desmosomes ++. Suprabasal differentiation.	43.0 ± 3.7*	Not assessed.	16.4 ± 19.1	16.1 ± 7.5	3.0 ± 1.4[$]	2.2 ± 0.8[$]
5% FBS KSFM (0.26 mM Ca)	Stratified. Tight junctions. No evidence of ATDL, but hyperproliferation of morphikens.	2567.1 ± 475**	Apical amiloride sensitive sodium channels. Basal Na-K-ATPase.	1.0 ± 1.4#	4.0 ± 5.4#	12.4 ± 7.6	5.5 ± 4.8

* significantly different ($p<0.01$) from 0.09 mM Ca KSFM
** significantly different ($p<0.01$) from 2mM and 0.09 mM Ca KSFM

ν Concentration of FITC-Dextran in the basal compartment, 3 hours after adding 1000 µg/ml FITC-Dextran to the apical chamber.
= Significantly different ($p<0.05$) from 0.09 mM Ca KSFM

$\perp$ significantly different ($p<0.01$) from 0.09 mM Ca KSFM and 5% FBS KSFM
No significant difference between 0.09 mM Ca KSFM and 5% FBS KSFM

Appendix B

The non-obvious nature of the invention for deriving biomimetic urothelium

Manuals detailing the culture of animal cells teach us that the dissociation of cells from a tissue and propagation on a 2D substrate leads to a loss of specialised features that often makes it difficult to relate the cultured cells to the functional cells in the tissue from which they were derived (Freshney 1987; p4). The culture environment is designed to "favour the spreading, migration and proliferation of unspecialised cells rather than the expression of differentiated functions" (Freshney 1987; p7). The medium used to provide nutrients to the culture is developed to provide optimal growth-promoting signals and "continuous proliferation may select undifferentiated precursors which, in the absence of the correct inductive environment, do not differentiate" (Freshney 1987; p10).

Proliferation-promoting conditions include the use of serum, growth factors and low cell density (Freshney 1987; Fig 2.3 p10). This latter is important as it illustrates that once a culture achieves confluence, cell proliferation becomes inhibited due to cell contact inhibition; to overcome this and promote further proliferation, cells are harvested from the substrate and redispersed (usually using a combination of trypsin in combination with EDTA) and reseeded at lower density in a sub-culture. "Serum has been retained for many cell types and is only gradually being eliminated after many years of careful and painstaking work" (Freshney 1987; p69). Selective media that avoid the use of serum have been developed for some cell types; keratinocyte serum free medium (KSFM) is one such serum-free medium which was developed originally for epidermal keratinocytes.

It is recognised that specific functions are retained for longer when the 3D structure of the tissue is retained, as in organ culture (Freshney 1987; p12) - e.g."Organ cultures cannot be propagated" - and so there are many attempts by cell biologists to recreate 3D tissue structures from propagated cells using a variety of methods (such as the use of scaffolds or matrices), which attempt to recapitulate the cell:cell and cell:matrix interactions of the originating tissue (Freshney 1987; p13).

From these points above, it is possible to conclude that:

1) The use of serum as a differentiating factor is non-obvious due to its association with promotion of proliferation.

2) The use of redispersal of cells as part of a differentiation-inducing procedure is non-obvious because such procedure:

(a) is routine in sub-culture to break cell:cell and cell:matrix contacts, to alleviate the contact inhibition of growth and to promote proliferation; and

(b) goes against the established approach of promoting 3D structure for achieving differentiation.

Reference

Freshney RI. Culture of animal cells: a manual of basic technique. 2nd edition 1987, Alan R Liss Inc.

Second Edition

CULTURE OF ANIMAL CELLS

A Manual of Basic Technique

R. Ian Freshney

Department of Medical Oncology
Cancer Research Campaign Laboratories
University of Glasgow

Alan R. Liss, Inc., New York

Cover Illustrations. *From the top: Vero cells growing on microcarriers; suspension culture vessels; primary explant from human mammary carcinoma; human glioma cells.*

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or interstitial fluid. This implies a level of skill and understanding to appreciate the requirements of the system and to diagnose problems as they arise. Tissue culture should not be undertaken casually to run one or two experiments.

Quantity

A major limitation of cell culture is the expenditure of effort and materials that goes into the production of relatively little tissue. A realistic maximum per batch for most small laboratories (two or three people doing tissue culture) might be 1–10 g of cells. With a little more effort and the facilities of a larger laboratory, 10–100 g is possible; above 100 g implies industrial pilot plant scale, beyond the reach of most laboratories, but not impossible if special facilities are provided.

The cost of producing cells in culture is about ten times that of using animal tissue. Consequently, if large amounts of tissue (>10 g) are required, the reasons for providing them by tissue culture must be very compelling. For smaller amounts of tissue (≤10 g), the costs are more readily absorbed into routine expenditure, but it is always worth considering whether assays or preparative procedures can be scaled down. Semimicro- or micro-scale assays can often be quicker due to reduced manipulation times, volumes, centrifuge times, etc. and are often more readily automated (see under Microtitration, Chapter 19).

Instability

This is a major problem with many continuous cell lines resulting from their unstable aneuploid chromosomal constitution. Even with short-term cultures, although they may be genetically stable, the heterogeneity of the cell population, with regard to cell growth rate, can produce variability from one passage to the next. This will be dealt with in more detail in Chapters 10 and 17.

MAJOR DIFFERENCES *IN VITRO*

Many of the differences in cell behavior between cultured cells and their counterparts *in vivo* stem from the dissociation of cells from a three-dimensional geometry and their propagation on a two-dimensional substrate. Specific cell interactions characteristic of the histology of the tissue are lost, and, as the cells spread out, become mobile and, in many cases, start to proliferate, the growth fraction of the cell population increases. When a cell line forms it may represent only one or two cell types and many heterotypic interactions are lost.

The culture environment also lacks the several systemic components involved in homeostatic regulation *in vivo*, principally those of the nervous and endocrine systems. Without this control, cellular metabolism may be more constant *in vitro* than *in vivo*, but may not be truly representative of the tissue from which the cells were derived. Recognition of this fact has led to the inclusion of a number of different hormones in culture media (see Chapter 7) and it seems likely that this trend will continue.

Energy metabolism *in vitro* occurs largely by glycolysis, and although the citric acid cycle is still functional it plays a lesser role.

It is not difficult to find many more differences between the environmental conditions of a cell *in vitro* and *in vivo* (see also Chapter 19) and this has often led to tissue culture being regarded in a rather skeptical light. Although the existence of such differences cannot be denied, it must be emphasized that many specialized functions are expressed in culture and as long as the limits of the model are appreciated, it can become a very valuable tool.

Origin of Cells

If differentiated properties are lost, for whatever reason, it is difficult to relate the cultured cells to functional cells in the tissue from which they were derived. Stable markers are required for characterization (see Chapter 13); and in addition, the culture conditions may need to be modified so that these markers are expressed (see next chapter and Chapter 14).

DEFINITIONS

There are three main methods of initiating a culture [Schaeffer, 1979] (see Glossary and Fig. 1.2): (1) *Organ culture* implies that the architecture characteristic of the tissue *in vivo* is retained, at least in part, in the culture (see Chapter 22). Toward this end, the tissue is cultured at the liquid gas interface (on a raft, grid, or gel) which favors retention of a spherical or three-dimensional shape. (2) In *primary explant culture* a fragment of tissue is placed at a glass (or plastic)/liquid interface where, following attachment, migration is promoted in the plane of the solid substrate (see Chapter 9). (3) *Cell culture* implies that the tissue, or outgrowth from the primary explant is dispersed (mechanically or enzymatically) into a cell suspension, which may then be cultured as an adherent monolayer on a solid substrate, or as a suspension in the culture medium (see Chapters 9 and 10).

Organ cultures, because of the retention of cell interactions as found in the tissue from which the

Chapter 2
Biology of the Cultured Cell

THE CULTURE ENVIRONMENT

The validity of the cultured cell as a model of physiological function *in vivo* has frequently been criticized. There are problems of characterization due to the alteration of the cellular environment; cells proliferate *in vitro* which would not normally *in vivo*, cell-cell and cell-matrix interactions are reduced because purified cell lines lack the heterogeneity and three-dimensional architecture found *in vivo*, and the hormonal and nutritional milieu is altered. This creates an environment which favors the spreading, migration, and the proliferation of unspecialized cells rather than the expression of differentiated functions. The provision of the appropriate environment, nutrients, hormones, and substrate is fundamental to the expression of specialized functions (see Chapter 14). Before considering such specialized conditions, let us examine the events accompanying the formation of a primary cell culture and a cell line derived from it (Fig. 2.1).

INITIATION OF THE CULTURE

Primary culture techniques are described in detail in Chapter 9. Briefly, a culture is derived either by outgrowth of migrating cells from a fragment of tissue, or by enzymatic or mechanical dispersal of the tissue. Regardless of the method employed, this is the first in a series of selective processes (Table 2.1) which may ultimately give rise to a relatively uniform cell line. In primary explantation (see Chapter 9) selection occurs by virtue of the cells' capacity to migrate from the explant, while with dispersed cells, only those cells which (1) survive the disaggregation technique and (2) adhere to the monolayer or survive in suspension will form the basis of a primary culture.

If the primary culture is maintained for more than a few hours, a further selection step will occur. Cells capable of proliferation will increase, some cell types will survive but not increase, and yet others will be unable to survive under the particular conditions used. Hence, the distribution of cell types will change and continue to do so until, in the case of monolayer cultures, all the available culture substrate is occupied. After confluence is reached (i.e., all the available growth area is utilized and the cells make close contact with one another), the proportion of density-limited cells gradually decreases, and the proportion of cells which are less sensitive to density limitation of growth (see Chapters 9 and 15) increases. Virally or spontaneously transformed cells will overgrow their normal counterparts. Keeping the cell density low, e.g., by frequent subculture, helps to preserve the normal phenotype in cultures such as mouse fibroblasts, where spontaneous transformants tend to overgrow at high cell densities [Todaro and Green, 1963; Brouty-Boyé et al., 1979, 1980].

Some aspects of specialized function are expressed more strongly in primary culture, particularly when the culture becomes confluent. At this stage the culture will show its closest morphological resemblance to the parent tissue.

EVOLUTION OF CELL LINES

After the first subculture—or passage (see Fig. 2.1)—the primary culture becomes a cell line (Chapter 10) and may be propagated and subcultured several times. With each successive subculture, the component of the population with the ability to proliferate most rapidly will gradually predominate, and nonproliferating or slowly proliferating cells will be diluted out. This is most strikingly apparent after the first subculture, where differences in proliferative capacity are compounded with varying abilities to withstand the trauma of trypsinization and transfer (see Chapter 10).

Although some selection and phenotypic drift will continue, by the third passage the culture becomes more stable, typified by a rather hardy, rapidly proliferating cell. In the presence of serum and without specific selection conditions (see Chapters 7 and 20)

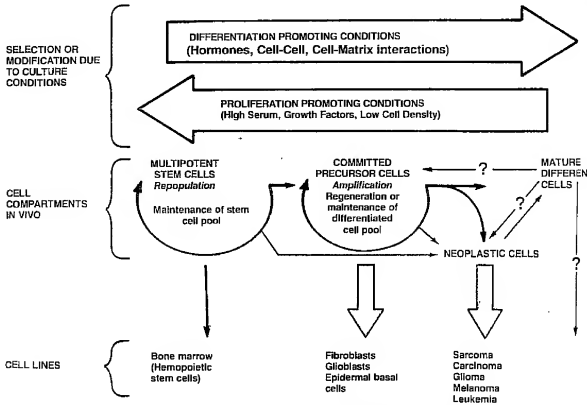

Fig. 2.3. *Origin of cell lines. With a few exceptions (e.g., differentiated tumor cells) culture conditions select for the proliferating cell compartment of the tissue or induce cells that are partially differentiated to revert to a precursor status. While neoplastic cells, and cell lines, may be derived from differentiated cells, it seems more likely that they arise from malignant precursor cells, some of which retain the capability to divide while continuing to differentiate.*

thereafter. Human glia [Pontén and Westermark, 1980] and chick fibroblasts [Hay and Strehler, 1967] behave similarly. Epidermal cells, on the other hand, have shown gradually increasing life-spans with improvements in culture techniques [Green et al., 1979] and may yet be shown capable of giving rise to continuous growth. This may be related to the self-renewal capacity of the tissue *in vivo* (see below, this chapter). Continuous culture of lymphoblastoid cell is also possible [Moore et al., 1967], although in this case, transformation with Epstein Barr virus may be implicated.

It is possible that the condition that predisposes to the development of a continuous cell line is inherent genetic variation, so it is not surprising to find genetic instability perpetuated in continuous cell lines. A common feature of many human continuous cell lines is the development of a subtetraploid chromosome number (see Fig. 2.2).

For a further discussion of variation and instability see Chapter 17.

DEDIFFERENTIATION

This implies that differentiated cells lose their specialized properties *in vitro*, but it is often unclear whether (1) undifferentiated cells of the same lineage (Fig. 2.3) overgrow terminally differentiated cells of reduced proliferative capacity or (2) the absence of the appropriate inducers (hormones: cell or matrix interaction) causes deadaptation (see Chapter 14). In practice both may occur. Continuous proliferation may select undifferentiated precursors, which, in the absence of the correct inductive environment, do not differentiate.

An important distinction should be made between dedifferentiation, deadaptation, and selection. Dedif-

Guguen-Guillouzo et al., 1983], hepatocytes can be selected preferentially. Similarly, epidermal cells can be grown either by using a confluent feeder layer [Rheinwald and Green, 1975] or selective medium [Peehl and Ham, 1980; Tsao et al., 1982]. The appearance of other examples, e.g., feeder selection for breast and colonic epithelium [Freshney et al., 1981], D-valine for the isolation of kidney epithelium [Gilbert and Migeon, 1975], and the use of cytotoxic antibodies [Edwards et al., 1980] (selection procedures reviewed in Chapters 11, 12, and 20), clearly demonstrate that the selective culture of specialized cells is not the insuperable problem that it once appeared.

WHAT IS A CULTURED CELL?

The question remains open, however, as to the exact nature of the cells that grow in each case. Expression of differentiated markers under the influence of inducing conditions may either mean that the cells being cultured are mature and only require induction to maintain synthesis of specialized proteins, or that the culture is composed of precursor or stem cells which are capable of proliferation but remain undifferentiated until the correct inducing conditions are applied, whereupon some or all of the cells mature to differentiated cells. It may be useful to think of a cell culture as being in equilibrium between multipotent stem cells, undifferentiated but committed precursor cells, and mature differentiated cells (see Fig. 2.3) and that the equilibrium may shift according to the environmental conditions. Routine serial passage at relatively low cell densities would promote cell proliferation and little differentiation while high cell densities, low serum, and the appropriate hormones would promote differentiation and inhibit cell proliferation.

The source of the culture will also determine which cellular components may be present. Hence cell lines derived from the embryo may contain more stem cells and precursor cells and be capable of greater self-renewal than cultures from adults. In addition, cultures from tissues which are undergoing continuous renewal *in vivo* (epidermis, intestinal epithelium, hemopoietic cells) will still contain stem cells, which, under the appropriate culture conditions, may survive indefinitely, while cultures from tissue which renew only under stress (fibroblasts, muscle, glia) may only contain committed precursor cells with a limited culture life-span.

Thus, the identity of the cultured cell is not only defined by its lineage *in vivo* (hemopoietic, hepatocyte, glial, etc.) but also by its position in that lineage (stem cell, committed precursor cell, or mature differentiated cell). With the exception of mouse teratomas and one or two other examples from lower vertebrates, it seems unlikely that cells will change lineage (transdifferentiate), but they may well change position in the lineage, and may even do so reversibly in some cases.

When cells are cultured from a neoplasm, they need not adhere to these rules. Thus a hepatoma from rat may proliferate *in vitro* and still express some differentiated features, but the closer they are to the normal phenotype, the more induction of differentiation may inhibit proliferation. Although the relationship between position in the lineage and cell proliferation may become relaxed (though not lost; B16 melanoma cells still produce more pigment at high cell density and at a low rate of cell proliferation than at a low cell density and a high rate of cell proliferation), transfer between lineages has not been clearly established (see also Chapter 14).

FUNCTIONAL ENVIRONMENT

Since the inception of tissue culture as a viable technique, culture conditions have been adapted to suit two major requirements: (1) production of cells by continuous proliferation and (2) preservation of specialized functions. The upsurge of interest in cellular and molecular biology and virology in the 1950s and 1960s concentrated mainly on fundamental intracellular processes such as the regulation of protein synthesis, often requiring large numbers of cells. Later, the development of such techniques as molecular hybridization and gene transfer allowed the emphasis to shift to the study of the regulation of specialized functions. While the need for bulk cultures remains, more attention has been directed to the creation of an environment which will permit the controlled expression of differentiation.

It has been recognized for many years that specific functions are retained for longer where the three-dimensional structure of the tissue is retained, as in organ culture (see Chapter 22). Unfortunately, organ cultures cannot be propagated, must be prepared *de-novo* for each experiment, and are more difficult to quantify than cell cultures. For this reason there have been numerous attempts to recreate three-dimensional structures by perfusing monolayer cultures [Kruse et al., 1970; Whittle and Kruse, 1973; Knazek et al., 1972; Knazek, 1974; Gullino and Knazek, 1979] and to reproduce elements of the environment *in vivo* by

culturing cells on or in special matrices like collagen gel [Michalopoulos and Pitot, 1975; Yang et al., 1981; Burwen and Pitelka, 1980], cellulose [Leighton, 1951] or gelatin sponge [Douglas et al., 1976], or matrices from other natural tissue matrix glycoproteins such as fibronectin, chondronectin, and laminin [Gospodarowicz et al., 1980; Kleinman et al., 1981; Reid and Rojkind, 1979] (see Chapter 7). These techniques present some limitations, but with their provision of homotypic cell interactions, cell matrix interactions, and the possibility of introducing heterotypic cell interactions, they may hold considerable promise for the examination of tissue-specific functions.

The development of normal tissue functions in culture would facilitate investigation of pathological behavior such as demyelination and malignant invasion. But, from a fundamental viewpoint, it is only when cells *in vitro* express their normal functions that any attempt can be made to relate them to their tissue of origin. Expression of the differentiated phenotype need not be complete, since the demonstration of a single cell type-specific cell surface antigen may be sufficient to place a cell in the correct lineage. More complete functional expression may be required, however, to place a cell in its correct position in the lineage, and to reproduce a valid model of its function *in vivo*.

TABLE 7.4. Balanced Salt Solutions

Component	Earle's balanced salt solution gm/l	Dulbecco's phosphate buffered saline (solution A) (PBSA) gm/l	Hanks' balanced salt solution gm/l	Spinner salt solution (Eagle) gm/l
Inorganic salts				
$CaCl_2$ (anhyd.)	0.02	—	0.14	—
$CaCl_2 \cdot 2H_2O$	—	—	—	—
KCl	0.04	0.20	0.40	0.40
KH_2PO_4	—	0.20	0.06	—
$MgCl_2 \cdot 6H_2O$	—	—	0.10*	—
$MgSO_4 \cdot 7H_2O$	0.20	—	0.10	0.20
NaCl	6.68	8.00	8.00	6.80
$NaHCO_3$	2.20	—	0.35	2.20
$Na_2HPO_4 \cdot 7H_2O$	—	2.16	0.09**	—
$NaH_2PO_4 \cdot H_2O$	0.14†	—	—	1.40
Other components				
D-glucose	1.00	—	1.00	1.00
Phenol red	0.01	—	0.01‡	0.01

*$MgCl_2 \cdot 6H_2O$ added to original formula.
†Original formulation calls for 150.0 mg/l $NaH_2PO_4 \cdot 2H_2O$. In Vitro, 9, #6 (1974).
**Original formulation calls for 0.06 gm/l $Na_2HPO_4 \cdot H_2O$. Proc. Soc. Exp. Biol. and Med., 71 [1949].
‡Original formulation calls for 0.02 gm/l. Proc. Soc. Exp. Biol. and Med., 71 [1949].

MEDIA AND SUPPLEMENTS

The discovery that cells from explants could be subcultured and propagated *in vitro* led to attempts to provide more defined media to sustain continuous cell growth and replace the "natural" media like embryo extract, protein hydrolysates, lymph, etc. Basal media of Eagle [1955, 1959] and the more complex media 199 of Morgan et al. [1950] and CMRL 1066 of Parker et al. [1957], although "defined", are usually supplemented with 5–20% serum; it was the desire to eliminate this remaining undefined constituent that led to the evolution of such complex media as NCTC 109, Evans et al. [1956], and 135, Evans and Bryant [1965], Waymouth's MB 572/1 [1959], Ham's F10 [1963] and F12 [1965], Birch and Pirt [1971], the MCDB series [Ham and McKeehan, 1978], and Sato's hormone-supplemented media [Barnes and Sato, 1980].

One approach to developing a medium is to start with a rich medium such as Ham's F12 [1965] or medium 199 supplemented with a high concentration of serum (say 20%) and gradually attempt to reduce the serum concentration by manipulating the concentrations of existing constituents and by adding new ones. This is a very laborious procedure but it has resulted in a number of different formulations for the culture of human fibroblasts and other cell types either in low serum concentrations or in its complete absence (see below).

Even after many years of exhaustive research into matching particular media to specific cell types and culture conditions, the choice of medium is not obvious and is often empirical. No all purpose medium has been developed for the more demanding requirements of specialized cells, and even transformed cells, cultured from spontaneous tumors, have highly specific requirements, differing among tumors, even of one type, and often differing from the normal cells of the same tissue. Hence serum has been retained for many cell types and is only gradually being eliminated after many years of careful and painstaking work.

PHYSICAL PROPERTIES

pH

Most cell lines will grow well at pH 7.4. Although the optimum pH for cell growth varies relatively little among different cell strains, some normal fibroblast lines perform best at pH 7.4–7.7, and transformed cells may do better at pH 7.0–7.4 [Eagle, 1973]. There have been reports that epidermal cells may be maintained at pH 5.5 [Eisinger et al., 1979]. It may prove advantageous to do a brief growth experiment (see Chapter 11 and 18) or special function analysis (e.g. Chapter 14) to determine the optimum pH.

Phenol red is commonly used as an indicator. It is red at pH 7.4, becoming orange at pH 7.0, yellow at pH 6.5, slightly bluish red at pH 7.6, and purple at pH 7.8. Since the assessment of color is highly subjective, it is useful to make up a set of standards using sterile balanced salt solution (BSS) and phenol red at the correct concentration and in the same type of bottle that you normally use for preparing medium.